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Preferential Species-Restricted Heavy/Light Chain Pairing in Rat/Mouse Quadromas

Implications for a Single-Step Purification of Bispecific Antibodies

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Conventional mouse/mouse or rat/rat hybrid-hybridoma supernatants contain up to 10 different IgG molecules consisting of various combinations of heavy and light chains. Hence, the yield of functional bispecific Ab is low, and purification is often complicated, hampering a general preclinical evaluation of, e.g., bispecific Ab-mediated tumor immunotherapy in animal models. In experiments to overcome this drawback we found that fusion of rat with mouse hybridomas opens the possibility of large scale production of bispecific Ab due to the increased incidence of correctly paired Ab and facilitated purification. In essence, rat/mouse quadroma-derived bispecific Ab have the following advantages: 1) enrichment of functional bispecific Ab because of preferential species-restricted heavy/light chain pairing (observed in four of four rat-mouse quadromas) in contrast to the random pairing in conventional mouse/mouse or rat/rat quadromas, and 2) a possible one-step purification of the quadroma supernatant with protein A. This simple chromatography step does not bind unwanted variants with parental rat/rat heavy chain configuration, and the desired rat/mouse bispecific Ab are retained, which can then easily be separated from parental mouse Ab by sequential pH elution. *The Journal of Immunology,* 1995, 155: 219–225.

Bispecific Abs (bsAb)[2] offer a variety of applications, including immunodiagnosis and therapy (1, 2). Various investigators have demonstrated in vitro and in vivo lysis of target cells by effector cells using bifunctional Ab (3–5). However, a general clinical evaluation as well as preclinical animal studies are hampered by certain disadvantages of the three methods presently used for the generation of bsAb. The chemical production of bsAb suffers from the different qualities of preparations after digestion, dissociation, and reassociation steps depending on the Ig subclasses (6, 7). Advances in the expression of Ig genes in *Escherichia coli* (8) and the recently reported success in producing a noncovalently linked bispecific Fv fragment in *E. coli* (9) have provided a possible solution to the problems of chemical heterogeneity by genetic techniques. However, because such molecules have no Fc portion and are not glycosylated, it is not yet clear whether recombinant Ab molecules produced by prokaryotes are comparable or even disadvantageous to normal Ab. bsAb assembled and secreted by the same process as native Ig can only be produced by the third method, the quadroma technology. The advantages resulting from this technique are 1) stability and pharmacokinetics comparable to those of normal Ab (10), and 2) practically unlimited production of bsAb by a defined clone. The main disadvantage is the difficult purification of the bispecific component from quadroma supernatants, which contain up to nine other Ab variants depending on the heavy/light chain combination.

Several investigations have dealt with this problem, and in some mouse/mouse quadromas, preferential pairing of certain chains was reported (11, 12). However, a statistically more relevant study with eight quadromas found that within 11 of the 16 described Ab specificities the H/L pairing were random (13). Therefore, in only a small number of cases will the "ideal" producer be met and, generally, the yield of bsAb in mouse/mouse quadromas is low. Here we report that in rat/mouse quadromas the H chains are reproducibly associated with L chains from the same species. As a consequence, the yield of functional bsAb is markedly increased. Furthermore, the fact that the rat H chains of a rat/mouse bsAb do not bind to protein A can be exploited for an easy single step purification of the desired bispecific component.

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[2] Abbreviation used in this paper: bsAb, bispecific antibodies.

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Table I. *Summary of established rat/mouse quadromas, their isotypes and specificities*

bsAb/ Quadroma	Hybridomas/ specificities	Species and IgG-isotypes	Origin
4C8	MmT1/2C4 Thy1.2/CD45R	m2a/r2b	AKR/J/Louvain (mouse/rat)
BiB	MmT1/RmCD8-6 Thy1.2/CD8	m2a/r2a	AKR/J/Louvain (mouse/rat)
BiC	HB3/17A2 I-A^d/CD3	m2a/r2b	B6xA/J-F1/Sprague-Dawley (mouse/rat)
G2	MmT1/17A2 Thy1.2/CD3	m2a/r2b	AKR/J/Sprague-Dawley (mouse/rat)

Table II. *Coating/detection-antibody combinations used for double (DIE) and single (SIE)-isotype ELISA*

ELISA-type	Coating/detection-antibody
DIE	anti rat 2b/anti rat 2b-biot.
	anti mouse 2a/anti mouse 2a-biot.
	anti rat 2b/anti mouse 2a-biot.
SIE	peak fract./anti mouse kappa-biot.
	peak fract./anti rat kappa-biot
	peak fract./anti rat 2b-biot.
	peal fract./anti bovine H+L HRP

Materials and Methods

Cells and media

The following parental hybridomas (see Table I) were used for quadroma production and have been described previously: MmT1 (mouse IgG2a, anti-mouse Thy1.2) (14), 17A2 (rat IgG2b, anti-mouse CD3) (15), RmCD8-6 (rat IgG2a anti-mouse CD8) (16), and HB3 (mouse IgG2a anti-mouse I-A^d) American Type Culture Collection (ATCC; Rockville, MD). The hybridoma 2C4 (rat IgG2b anti-mouse CD45R) was generated and characterized in our laboratory. BCL1 lymphoma cells expressing I-A^d (MHC class II) (kindly provided by Dr. Thielemanns, Brussels, Belgium) (17) and CBA spleen cells from mice raised under SPF conditions in our institution were used as target cells for FACS analysis. Cells were cultured in RPMI 1640 containing 10% FCS supplemented with antibiotics and L-glutamine.

Production of quadromas

Quadromas were produced as previously described (18). Briefly, one partner for the fusion was selected for the loss of the HPRT+ phenotype by growth in the selective drug 8-azaguanine. The other fusion partner was a conventional HAT-resistant hybridoma cell line. HAT-resistant cells (5×10^6) were washed with PBS and then resuspended in 10 ml of PBS at 4°C containing 2.5 to 5 mM iodoacetamide. After 30 min, the cells were washed three times with culture medium and mixed with 1.5 $\times 10^7$ HAT-sensitive cells. The cells were pelleted at $200 \times g$, and cell fusion was induced by treating the cell pellet for 2 min with 1 ml of polyethylene glycol 1500 while stirring. The cells were then plated out into eight 96-well culture plates. Selection with HAT-containing medium was started on the second or third day after fusion.

Purification of rat/mouse bsAb on protein A

To isolate hybrid Ab molecules of the subclass combinations rat IgG2b/mouse IgG2a and rat IgG2a/mouse IgG2a from quadroma supernatants, the supernatants were centrifuged, filtered, and loaded onto a 5-ml Econo Pac protein A column (Bio-Rad, Richmond, CA). After washing with 10 bed volumes of PBS, Ab with the hybrid H chain configuration were eluted with 0.1 M citric acid at pH 5.8. Subsequently, parental mouse Ab were eluted by 0.1 M citric acid, pH 3.5, thereby regenerating the column. Finally, the column was re-equilibrated with PBS containing 0.04% sodium azide.

FPLC analysis of bsAb

Protein A and protein G fractions were subjected to FPLC cation exchange chromatography on a Mono S column (Pharmacia, Uppsala, Sweden). Samples were dialyzed against 40 mM MES buffer (pH 5.1) before application. Ab were eluted from the column by four consecutive linear gradients, ranging from 0 to 120, 120 to 210, 210 to 450, and 450 to 800 mM NaCl in MES buffer. The elution profile was determined by monitoring the absorbance at 280 nm.

ELISA

The following subclass- and species-specific monoclonal and polyclonal detection Ab were used: TIB169 (mouse IgG2b anti-rat κ, ATCC), TIB174 (mouse IgG2b anti-rat IgG2b, ATCC), R12-4 (rat IgG2a anti-mouse IgG2a, PharMingen), HB58 (rat IgG1 anti-mouse κ, ATCC), goat anti-bovine H+L (Kirkegaard & Perry, Gaithersburg, MD). All Ab were used directly, biotinylated or HRP conjugated.

Double isotype ELISA. ELISA plates were coated overnight with 5 μg/ml capture Ab. After washing, plates were blocked with 1% milk powder for at least 1 h. Subsequently, supernatants or chromatography fractions were incubated. Bound Ab was detected with the use of a biotinylated, second anti-isotypic Ab, followed by streptavidin-peroxidase. Finally, the substrate *ortho*-phenylenediamine was added, and absorbance was determined at 405 nm.

Single isotype ELISA. ELISA plates were coated overnight with titrated Mono S peak fractions. The presence of Ig chains was determined by second detection Ab. All coating/detection-antibody combinations are listed in Table II.

Quantification of parental Ab, bsAb, and mismatches. To quantify parental Ab, bsAb, and mismatches within the different Mono S peak fractions, we first adjusted the working dilutions of the detection Ab TIB169, TIB174, R12-4, and HB58 with purified functional bsAb. Subsequently, a single isotype ELISA was performed, as described above. By comparison of titers determined with the different second Ab, we calculated the distribution of parental Ab, bsAb, and mismatch types 1, 2, 6, and 7 (Fig. 1) for the different Mono S peak fractions.

Immunoblotting

Peak fractions of Mono S chromatography were separated on ExcelGel 8 to 18% SDS-polyacrylamide gels (Pharmacia). Immunostaining of the blotted proteins was performed with rat anti-mouse H+L chain, mouse anti-rat κ-chain HRP-conjugated polyclonal antiserum (Dianova, Germany) and TIB 174 mouse anti-rat IgG2b biotinylated mAb (ATCC) followed by streptavidin-peroxidase (Amersham International, Amersham, UK).

FACS

Flow cytometric experiments were performed using a FACScan (Becton Dickinson, Rutherford, NJ). For indirect immunofluorescence staining, 2.5×10^5 target cells were washed with FACS buffer (PBS, 1% NaN_3, and 1% FCS) and resuspended in 50 μl of the first stage Ab (Mono S peak fraction) for 30 min at 4°C. After washing, cells were resuspended in 50 μl of the appropriately diluted fluorescence-conjugated second Ab for an additional 30 min at 4°C. Dead cells were stained by propidium iodide.

Results

Principle of the single step purification

We have produced four rat/mouse quadromas whose specificities and isotypes are compiled in Table I. For large scale production, which is a prerequisite for application in vivo, we devised a single step purification procedure, which is exemplified below with the bsAb BiC. Figure 1

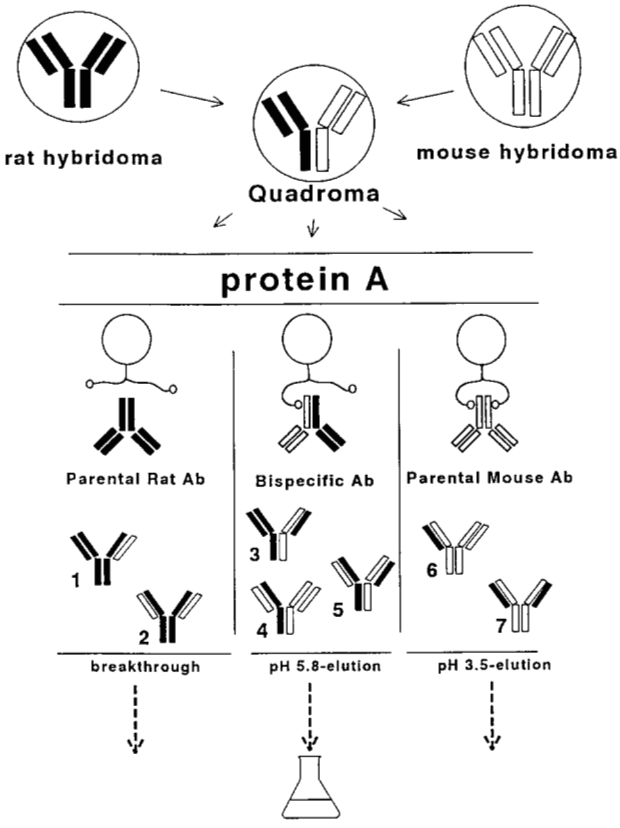

FIGURE 1. Single-step purification principle for rat/mouse quadroma-derived bispecific Ab. Circles in the center of the figure represent carrier beads with covalently linked protein A-binding domains. Mismatch variants are indicated by numbers.

schematically depicts the separation principle. Most importantly, parental rat Ab and undesired variants with homologous rat H chain configuration will not bind to protein A. At the same time, contaminating bovine Ig is also removed in the breakthrough fraction, because it does not bind to protein A at neutral pH. Deisenhofer (19) has shown that one protein A-binding domain (of five domains in a complete 42-kDa protein A molecule) binds between the CH2 and CH3 regions of one H chain. Therefore, two such binding domains usually bind to the complete Fc region of a parental mouse Ab. As the rat IgG2b H chain has no significant affinity to protein A, only one protein A-binding domain binds to the mouse portion of the heterologous molecule. Because of the resulting different affinities, rat/mouse-bispecific Ab can then be easily eluted at a near-physiologic pH of 5.8, whereas parental mouse Ab are stably retained in the column (Fig. 1). The column can be regenerated by eluting the parental mouse Ab at pH 3.5.

Single step purification of the bsAb BiC on protein A

To isolate hybrid Ab molecules, the quadroma supernatant was loaded onto a protein A column. Hybrid molecules consisting of heterologous heavy chains (verified by a double isotype ELISA) could be quantitatively adsorbed because no further pH 5.8 elution peak was found in a second loading/elution cycle (Fig. 2). The parental mouse Ab were recovered only at pH 3.5. To verify the prediction that rat IgG2b and bovine Ig do not bind to protein A at neutral pH (our own experiments and reference 20), we loaded the protein A breakthrough fraction onto a protein G column for further analysis. In the protein G pH 2.7 eluate, we found the expected parental rat Ab as well as bovine Ig. The identity of the peaks eluted from protein A and protein G was verified by subsequent cation exchange chromatography (Fig. 3), immunoblotting (Fig. 4), ELISA, and FACS.

Analysis of the protein A and G fractions of the bsAb BiC by cation exchange chromatography, ELISA, immunoblotting, and FACS

The peaks shown in Figure 2 were analyzed by double isotype ELISA and assigned as indicated. We further examined the fractions with a Mono S cation exchange column to evaluate the extent of heterogeneity in these fractions (Fig. 3). The parental Ab involved in the four bsAb combinations used in this study (Table I) displayed different ionic properties, allowing a cation exchange analysis. Coelution of L chain mismatches in the different fractions, however, could not be excluded. To detect such L chain mismatches, we subsequently analyzed the Mono S peak fractions by ELISA, immunoblotting, and FACS.

In many cases, a serologic detection system for different mouse κ L chains is not available. Therefore, mismatched L chains in mouse/mouse quadromas are often detectable only by molecular mass differences of the involved L chains. In contrast, in rat/mouse quadromas, the different light chains can easily be detected immunologically with high sensitivity. This advantage of rat/mouse quadromas was exploited for the detection of possible mismatch variants. To detect mismatched L chains with high sensitivity, we focused on the protein A/G fractions with the homologous mouse H chain configuration (protein A at pH 3.5) and the homologous rat H chain configuration (protein G at pH 2.7). We assumed that if the H/L chain pairing in rat/mouse quadromas is at random, as in mouse/mouse quadromas, then 50% of the L chains in the protein A pH 3.5 fraction should be of rat origin. The same should be true for mouse L chains in the protein G pH 2.7 fraction (see Fig. 1). Such mismatches would be easily detectable in immunoblots.

Figure 3 shows representative FPLC separations of the protein A and protein G fractions for the bsAb BiC. The elution profile of the pH 5.8 protein A fraction is shown in section A. The existence of only one main peak suggests a high purity of the bsAb. Immunologic assays, such as ELISA, immunoblotting (Fig. 4), and FACS (Table III), confirmed the bispecific character of this peak. The minor

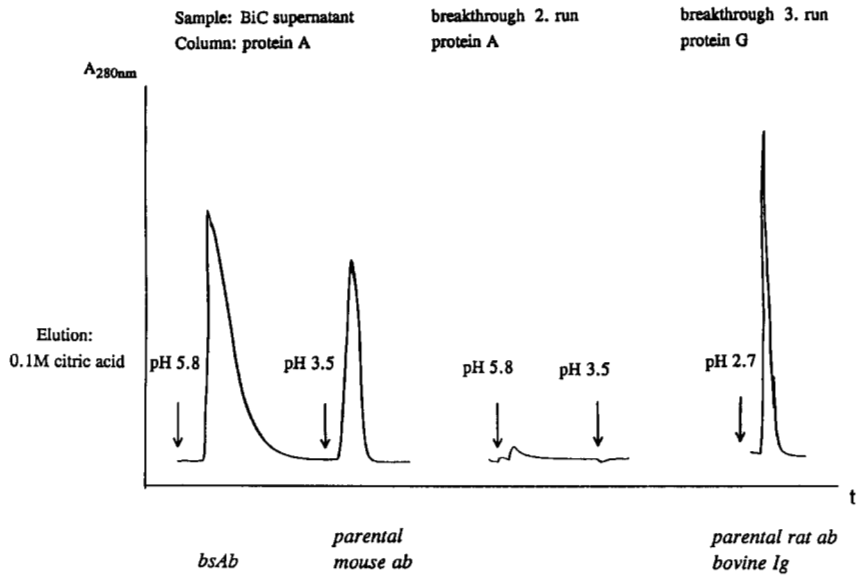

FIGURE 2. Single-step purification of the bsAb BiC on protein A. Peaks were eluted using step gradients. The figure represents the absorbance (280 nm) vs time.

peak 1 was a candidate for a possible mismatch variant; by immunoblotting and ELISA, however, we found that it probably contains a differently glycosylated rat H chain within the bsAb (Fig. 4), parental mouse Ab, and mismatch types 3, 6, and 7. By comparison of titers obtained by ELISA and FACS with the relevant second Ab, we calculated a composition of about 50% parental mouse Ab, 20% bsAb, and 30% mismatches for this peak. Based on the protein amounts in peaks 1 and 2 (Fig. 3*A*), the main contamination within the protein A, pH 5.8 (bsAb), fraction was calculated to be 5% parental mouse Ab and mismatch types 3, 6, and 7. Compared with the bsAb peak fraction (Fig. 3*A*, peak 2), the parental mouse Ab (Fig. 3*B*, peaks 1 and 2) appeared earlier using this particular salt gradient. ELISA, immunoblotting, and FACS analyses revealed a contamination with bsAb in these peaks 1 and 2. By comparison of ELISA and FACS titers, we calculated a composition of 94% parental mouse Ab, about 3% bsAb, and 3% mismatches of types 6 and 7 (Fig. 1) for this peak. Peak 3 (Fig. 3*B*) could be characterized as residual bsAb by immunoblotting (Fig. 4) and ELISA. Figure 3*C* shows the elution profile of the pH 2.7 protein G fraction representing the Ig in the breakthrough of the protein A column. It contains parental rat Ab (peaks 1 and 2) and bovine Ig (mainly within peak b) distributed over the whole range of this run, as evaluated by ELISA and immunoblotting (Fig. 4). Mismatched mouse L chains to rat H chains could be detected in only trace amounts in peak 2 by ELISA.

By immunoblotting, no mismatched mouse L chains were visible in peaks 1 and 2 (pH 2.7), indicating the rare incidence of this association event (Fig. 4*A*). Immunoblotting also revealed the occurrence of two types of rat H chains varying in molecular size. As the small H chain, representing about 94% (determined by comparing the bsAb protein amounts of peak 2, *A*, and peak 3, *B*, with

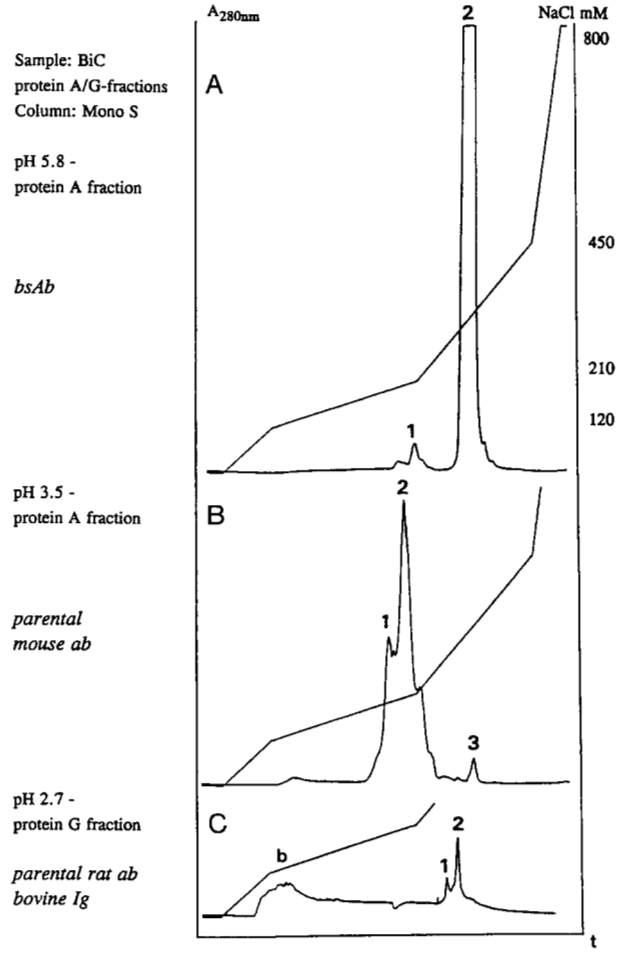

FIGURE 3. Elution profiles of protein A and G fractions of bsAb BiC on cation exchange FPLC (Mono S column). The figure represents the absorbance (280 nm) and salt gradient steps vs time. Numbered peaks were examined by ELISA, immunoblotting, and FACS. Peak b, bovine Ig.

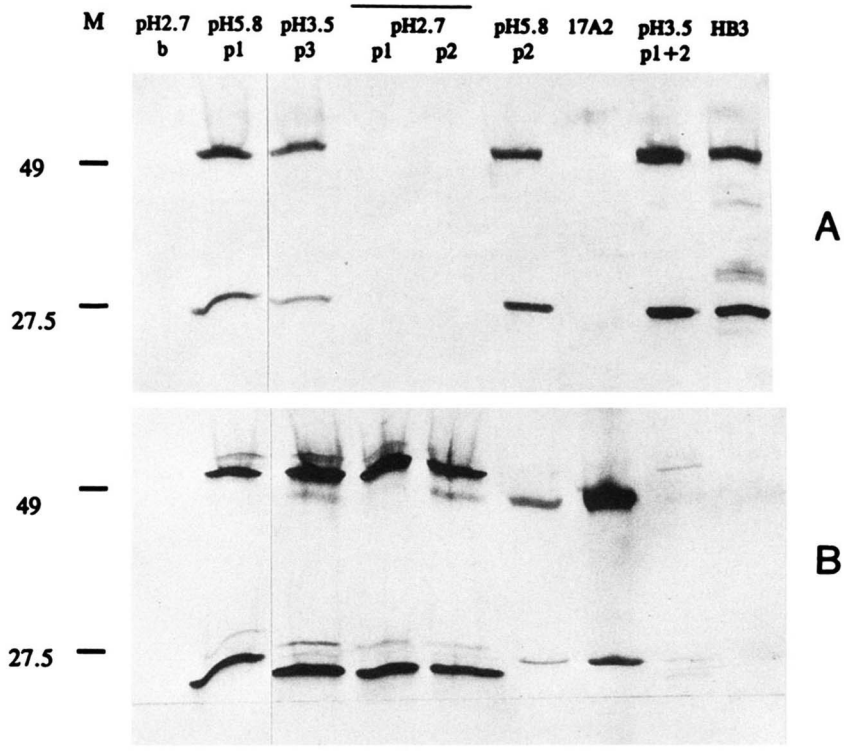

FIGURE 4. Immunoblot analysis of elution profiles of protein A and G fractions of bsAb BiC on cation exchange FPLC (Mono S). Mono S peak fractions were collected and immunologically evaluated for heavy/light chain composition and species origin. Each *lane* contains approximately 4 μg of the peak fraction indicated and control Ab 17A2 (rat IgG2b) and HB3 (mouse IgG2a). *A*, anti-mouse immunoblot. *B*, Anti-rat immunoblot. Rat L chain double and triple bands are due to reduction artifacts.

Table III. *Reactivity of Mono S fractions with target cells determined by FACS*

Target antigen:	I-A^d on BCL1		CD3 on CBA spleen	
Detection antibody:	α mouse IgG2a	α rat IgG2b[a] (bsAb only)	α rat IgG2b	α mouse IgG2a[a] (bsAb only)
pH5.8 peak 1	1/128	<1	1/2	1/8
pH5.8 peak 2	1/32	1/128	1/75	1/128
pH3.5 peak 1+2	1/256	–	1	1/4
pH3.5 peak 3	n.d.	n.d.	n.d.	n.d.
pH2.7 peak 2	–	–	1/256	–

All samples were adjusted to 15 μg/ml. Data are titers at half maximal fluorescence intensity.

–, Denotes a mean fluorescence less than 5% above background; n.d., not done.

[a] Antibodies detect in this configuration only bsAb.

The negative result of pH 3.5 peak 1+2 with detection antibody rat IgG2b does not preclude the presence of functional bsAb since the at least 10-fold excess of parental HB3 antibody could have prevented the single-arm binding of bsAb.

peak 1, *A*; peaks 1 and 2, *B*; and parental rat peaks 1 and 2, *C*) of rat H chains, eluted later in the salt gradient we used (Fig. 3, *A* and *B*) than the large H chain, one can suppose a different total charge of these molecules. A possible explanation for this observation may be a known microheterogeneity in glycoproteins due to different sialic acid content.

As immunoblot analysis cannot discriminate between a mismatch of type 5 (Fig. 1) and functional bsAb, we tested the Mono S fractions for their ability to bind both target Ag. The results are shown in Table III.

Production of bsAb, parental Ab, and mismatch variants of the four tested quadromas

Analysis of the protein A eluates by cation exchange chromatography and subsequent immunologic assays was performed for all four quadromas as described above for the bsAb BiC. It revealed a clear underrepresentation of mismatch variants for all four tested quadromas compared with a conventional mouse/mouse quadroma (Fig. 5). The ratios given for the conventional mouse/mouse quadroma are those expected for a completely random H/L and H/H chain pairing (21). Moreover, in all clones we observed a lower production of rat Ig than mouse Ig. The yield of bsAb per l supernatant is indicated in Fig. 5.

Discussion

A general evaluation of bispecific Ab in preclinical animal models is hampered by the difficulty of producing sufficient amounts, mainly because of two drawbacks: 1) the cumbersome purification of the bispecific component from quadroma supernatants, and 2) the low yield of bsAb produced by mouse/mouse or rat/rat quadromas due to a high rate of H/L mismatches. As a possible solution to these problems, we propose the use of rat/mouse quadromas. Analysis of the protein A eluates by cation exchange chromatography and subsequent immunologic assays revealed a substantial decrease in mismatch variants for all four tested rat/mouse quadromas (Fig. 5). In the case of bsAb BiC, the low amount of mismatched mouse L chains in the

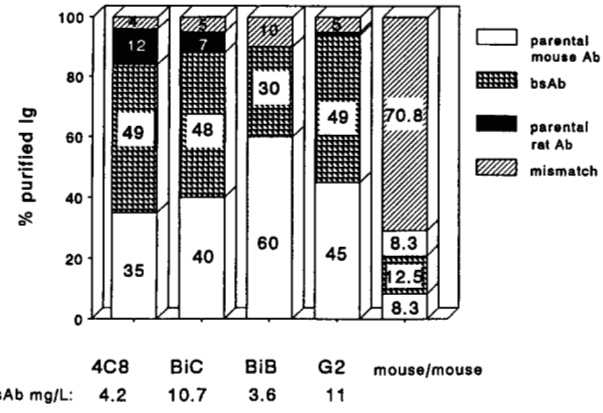

FIGURE 5. Bispecific Ab production of rat/mouse quadromas. The ratios (percentages) were calculated after characterization of all cation exchange chromatography peak fractions of the indicated bsAb (as exemplified with BiC) and determination of their protein content. mouse/mouse, Theoretical ratio of parental mouse Ab, bsAb, and mismatch variants in a mouse/mouse quadroma with nonrestricted H/L and H/H pairing (21).

parental rat peaks (pH 2.7 peaks 1 and 2), where mismatches are easily detectable, clearly demonstrated a restricted H/L pairing within the rat portion of the bsAb. A comparably clear result from the parental mouse Ab (pH 3.5 peaks 1 and 2) fraction was not possible because of a slight contamination with bsAb (Fig. 4 and Table III). However, ELISA and immunoblotting showed that the ratio between rat and mouse L chains strongly deviated from a 1:1 distribution, as expected for random pairing of H and L chains (see Fig. 1). Conversely, only about 6% of total L chains were of rat origin within this parental mouse Ab peak. Subtracting the bsAb from that 6%, only 3% remain as mismatches of type 6 and 7 (Fig. 1). As we detected mismatch types 6 and 7, we consider the existence of mismatch type 3 in this range of bsAb peak fractions to be possible. From these data we estimate that mismatch variants comprise about 5% of the total BiC quadroma Ab production. Titrating the binding activity of each arm of bsAb on cells expressing only one of the two Ag indicated no significant differences in binding activity of both binding arms (pH 5.8 peak 2; Table III). These FACS data suggest that the calculated amount of mismatches within the bsAb peak is correct in magnitude.

As shown in Figure 5, the other rat/mouse quadromas displayed a similar ratio of functional bsAb, parental Ab, and mismatch variants as quadroma BiC. One can conclude that rat/mouse quadromas have, on the average, a 3.5-fold higher production of bsAb compared with conventional mouse/mouse or rat/rat quadromas. The finding that preferential pairing was observed in different strain combinations (Table I) suggests that homologous H/L association may not be due to allotypic differences and represents a common principle.

The H/H pairing is obviously not affected in the isotype combinations used, because a hybrid H/H configuration was in no case diminished (Fig. 5). A possible explanation for this finding could be the sequence homology between isotypes rat IgG2b and mouse IgG2a (22) allowing a free H chain recombination. Conversely, two reports observed an enhanced preferential homologous H/H chain association when using the mouse isotype combination IgG1/IgG2a in quadromas (21, 23). Similarly, we observed a strongly diminished hybrid H/H association in a quadroma generated by fusion of two hybridomas that have been gene targeted (24) to express the human IgG1 and IgG3 isotypes, respectively. We assume that the diverging structure of hinge regions of certain isotypes (e.g., the different number of cysteines, two in human IgG1 and 15 in human IgG3) could be one reason for this observation.

However, if the incompatible hinge regions are suitably adapted, e.g., by exchange of the IgG3 hinge with an IgG1 hinge region, the same concept for the purification of bsAb as that used for rat/mouse quadromas may be employed with humanized Ab. This is possible because human IgG1 binds to protein A, and IgG3 does not.

The second aspect, i.e., large scale generation of bsAb, which is also a prerequisite for clinical use, depends on the availability of a simple purification procedure. An easy single step purification on protein A reflects a further advantage of rat/mouse bsAb. The purification of bsAb on protein A by sequential pH elution was first demonstrated by Couderc and colleagues (25). In those experiments the bispecific component was separated by exploiting the different affinities of mouse subclasses IgG1 and IgG2a to protein A. As protein A binds to certain areas of the CH2 and CH3 regions of the H chain (19), it could not discriminate between possible H/L mismatches. Therefore, the benefit of this purification method was small in the case of mouse/mouse quadromas, which, in general, do not exhibit preference for homologous H/L chain association (13, 26). Another disadvantage of mouse IgG1/2a bsAb is the occurrence of impurities in the protein A elution fractions. As the elution buffers for bsAb and parental Ab differ by as little as 0.5 pH units, the resolution of this method is hardly satisfactory. In the case of rat/mouse bsAb, parental rat Ab (with the exception of isotype IgG2c) do not bind to protein A at neutral pH, thus already reducing this problem. Moreover, rat/mouse bsAb elute from protein A at pH 6, probably due to the absence of any contributing binding affinity of the rat portion to protein A. On the contrary, mouse IgG1/2a bsAb begin to elute only at pH 5.5 to 5.0, possibly due to weak binding of the mouse IgG1 H chain and, thus, a higher total affinity to protein A.

A general problem of quadroma technology is the instability of clones, i.e., the loss of single H or L chains. Therefore, quality control is an important aspect pointing to another advantage of rat/mouse bsAb: the feasibility of fast immunologic screening, especially of the L chains. In the case of mouse κ-chains, which do not differ by m.w.,

testing for a loss of L chains could be a problem or even impossible for mouse/mouse bsAb. A more practical but relevant aspect is the saving of theoretically one third of capacity on the protein A column compared with the purification of, e.g., mouse IgG1/2a bsAb (where both isotypes bind to protein A). Taken together, all discussed advantages of rat/mouse quadromas may facilitate a further evaluation of possible curative therapies by bsAb.

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